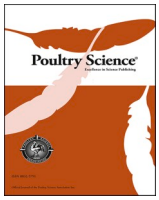
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Effects of decreasing phosphorus concentrations in diets and phytase supplementation on growth performance, stress response, and intestinal health in broiler chickens

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ABSTRACT

The current study aimed to investigate the effect of decreasing P concentrations in diets and phytase supplementation on growth performance, stress response, and intestinal health in broiler chickens. A total of four hundred 8-d-old Ross broiler chicks were randomly allotted to 1 of 4 dietary treatments with 10 replicates. Three treatment diets were formulated to contain different concentrations of available P (**aP**): adequate P (0.44 % aP), low P (0.30 % aP), and very low P (0.10 % aP) levels. One additional diet was prepared by supplementing 1,000 FTU/kg phytase in very low-P diets to maintain its aP concentrations equal to those in low-P diets. The experiment lasted for 25 d. It was observed that growth performance and toe ash concentrations were decreased ($P < 0.05$) in birds fed very low-P diets compared with those fed other treatment diets. Birds fed very low-P diets exhibited a greater ($P < 0.05$) blood heterophil:lymphocyte ratio than those fed other treatment diets. Jejunal villus height in birds fed adequate P diets or low-P diets was greater ($P < 0.05$) than those fed very low-P diets or very low-P diets with phytase. The malondialdehyde concentrations in the jejunal mucosa were greater ($P < 0.05$) for very low-P treatment than for other treatments. The expression levels of *IL-4* and *OCLN* in the jejunal mucosa were less ($P < 0.05$) for adequate P treatment than for low-P treatment and very low-P with phytase treatment. Expression levels of P transporter genes in the jejunal mucosa were not affected by dietary treatments. In conclusion, feeding very low-P diets impaired growth performance, bone development, and jejunal morphology in broiler chickens with elevated stress response and intestinal lipid peroxidation. However, feeding low-P diets or very low-P diets with phytase maintained bone development, stress response, and antioxidant status in broiler chickens. The gene expression of intestinal P transporters were not influenced by decreasing P concentrations in diets and phytase supplementation in very low-P diets under the current experimental condition.

Introduction

Phosphorus (**P**) is an essential macromineral involved in various body functions, including bone development, energy production, nucleic acid synthesis, and acid-base balance in poultry (NRC, 1994). Consequently, inadequate supply of dietary P can lead to an impairment in productive performance, product quality, and health in poultry, particularly during the rapid production period (Atia et al., 2000). Therefore, poultry nutritionists invest considerable efforts to formulate feeds with a sufficient amount of available P (**aP**) to ensure the optimal production and health in poultry. However, increasing P concentrations in poultry feed formulation is limited by high costs of dietary P sources as well as increasing concerns on environmental pollution (Cardoso et al., 2018).

In a practical condition, therefore, dietary P concentrations are tightly controlled at the level approximating its recommendation. Moreover, dietary phytase is routinely supplemented to poultry diets for improving dietary P utilization (Adeola and Cowieson, 2011). However, these nutritional approaches may unexpectedly lower P concentrations in diets as compared to their recommended levels owing to an inaccurate estimation of P availability in feed ingredients and phytase activity. In addition to possible suboptimal P supply in diets, dietary P deficiency of poultry may also be facilited by potential animal and environmental factors increasing P requirement. For example, the current poultry

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breeds exhibit a higher productive performance than previous breeds as a result of intensive genetic selection, leading to an increase in P requirement (McDevitt et al., 2006; Tischler et al., 2013). Moreover, various stressful environments, such as heat stress and high stocking density, may also increase P requirement (Lee et al., 2019; Sun et al., 2018). Consequently, if increasing P requirement of poultry is accompanied with an insufficient P supply in diets, poultry may suffer from P deficiency. Several previous studies have examined the effect of decreasing P concentrations in diets; however, most of these studies have emphasized on bone development and function in poultry (Li et al., 2020; Valable et al., 2018). Few studies have investigated the effect of decreasing P concentrations in diets and phytase supplementation in low-P diets on stress response and intestinal health in broiler chickens. Therefore, it may be hypothesized that decreasing P concentrations in diets enhance stress response and impair intestinal health in broiler chickens and these negative outcomes are mitigated by dietary phytase supplementation. However, this hypothesis has not been tested previously.

Therefore, the current study aimed to investigate the effect of decreasing P concentrations from adequate P to very low P levels in diets and phytase supplementation in very low-P diets on growth performance, stress response, and intestinal health in broiler chickens.

Materials and methods

All experimental procedures in the current study were reviewed and approved by the Animal Care and Use Committee at Chung-Ang University.

Animals, experimental design, and treatment diets

A total of four hundred 8-d-old Ross 308 broiler chicks (200 male and 200 female chicks) with similar body weight (**BW**; initial BW = 204 ± 8.1 g) were allotted to 1 of 4 dietary treatments with 10 replicates in a completely randomized design. Each replicate consisted of 5 male and 5 female birds. Before the start of the experiment, all birds were fed a commercial starter diet containing 3,000 kcal/kg AME_n, 23.0 % CP, 1.30 % digestible Lys, 1.0 % digestible Met+Cys, 1.0 % total Ca, and 0.50 % aP. All birds were housed in battery cages (76.0 × 78.0 × 45.0 cm, width × length × height) for 25 d of the feeding trial. The room temperature was maintained at 28°C at the start of the experiment and then gradually decreased to 20°C as recommended by the Ross 308 broiler management guidelines (Aviagen, 2018). A 23-h lighting schedule was applied during the entire experimental period.

Three treatment diets were formulated to contain different concentrations of aP (Table 1). An adequate P diet was formulated to contain

Table 1
Composition and nutrient concentrations of the experimental diets.

Items	Dietary treatments[a]			
	Adequate P	Low P	Very low P	Very low P + phytase
Ingredients (%)				
Corn	50.60	50.60	50.60	50.60
Soybean meal (45 % CP)	35.00	35.00	35.00	35.00
Corn gluten meal	2.50	2.50	2.50	2.50
Tallow	6.46	6.46	6.46	6.46
Limestone	1.94	1.60	1.60	1.60
MSP[b] (25 % available P)	1.34	0.80	0.00	0.00
Phytase[c]	0.00	0.00	0.00	0.0167
Celite	0.32	1.20	2.00	1.9833
DL-Met (99 %)	0.34	0.34	0.34	0.34
L-Lys-HCl (99 %)	0.38	0.38	0.38	0.38
L-Thr (98 %)	0.06	0.06	0.06	0.06
NaCl	0.40	0.40	0.40	0.40
Coccidiostats	0.12	0.12	0.12	0.12
$NaHCO_3$	0.14	0.14	0.14	0.14
Antioxidant	0.10	0.10	0.10	0.10
Vitamin premix[d]	0.15	0.15	0.15	0.15
Mineral premix[e]	0.15	0.15	0.15	0.15
Total sum	100.00	100.00	100.00	100.00
Calculated energy and nutrient[f]				
AME_n (kcal/kg)	3,100	3,100	3,100	3,100
CP (%)	21.60	21.60	21.60	21.60
Total Lys (%)	1.30	1.30	1.30	1.30
Total Met + Cys (%)	1.00	1.00	1.00	1.00
Total Thr (%)	0.88	0.88	0.88	0.88
Total Trp (%)	0.26	0.26	0.26	0.26
Total Ca (%)	0.88	0.76	0.76	0.76
Total P (%)	0.69	0.56	0.36	-
Available P (%)	0.44	0.30	0.10	0.30
Phytate (%)	0.26	0.26	0.26	0.26
Analyzed CP (%)	21.31	21.23	20.77	21.64
Analyzed total Ca (%)	0.87	0.75	0.76	0.77
Analyzed total P (%)	0.71	0.55	0.35	0.36

[a] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase.

[b] MSP, monosodium phosphate.

[c] *Escherichia coli*-derived phytase (6,000,000 FTU/kg) was used and P equivalency value for 1,000 FTU/kg phytase was assumed to be 0.20 % available P.

[d] Provided per kg of the complete diet: vitamin A, 12,000 IU (retinyl acetate); vitamin D_3, 4,000 IU; vitamin E, 80.0 mg; vitamin K_3, 4.0 mg (menadione dimethylpyrimidinol); vitamin B_1, 4.0 mg; vitamin B_2, 10.0 mg; vitamin B_6, 6.0 mg; vitamin B_{12}, 20.0 μg; folic acid, 2.0 mg; biotin, 200 μg; niacin, 60 mg.

[e] Provided per kg of the complete diet: iron, 60 mg ($FeSO_4$); zinc, 100 mg ($ZnSO_4$); manganese, 120 mg (MnO); copper, 16 mg ($CuSO_4$); cobalt, 1,000 μg ($CoSO_4$); selenium, 300 μg (Na_2SeO_3); iodine, 1.25 mg [$Ca(IO_3)_2$].

[f] Calculated energy and nutrient concentrations were based on CVB (2022).

0.44 % aP as recommended by the Ross 308 broiler management guidelines (Aviagen, 2019), whereas a very low-P diet was formulated to contain 0.10 % aP with no inclusion of inorganic P supplements. A low-P diet containing 0.30 % aP was also prepared by adding 0.8 % monosodium phosphate (**MSP**; 25 % aP) to very low-P diets. Finally, an additional diet was prepared by supplementing 1,000 FTU/kg phytase (6,000,000 FTU/kg *Escherichia coli*-derived phytase) to very low-P diets. The calculated concentration of aP in very low-P diets with phytase was set at 0.30 % aP, which was equalized to aP concentrations in low-P diets by applying assumed equivalency value of aP for phytase (i.e., 1,000 FTU/kg phytase = 0.20 % aP). The MSP and phytase were supplemented to very low-P diets in replace of celite, respectively. The calculated concentration of total Ca in all 3 low-P diets was 0.76 %, which was established based on 14.0 % reduction of total Ca concentration in adequate P diets (0.88 %) by reason of minimizing the negative effect of a large variation in dietary Ca:aP ratio (Kwon et al., 2022). The calculated concentrations of energy and nutrients other than total Ca and aP were equalized among all treatment diets. The analyzed concentrations of total CP, Ca, and P in treatment diets were close to their calculated concentrations (Table 1). Experimental diets were prepared in mash form. The diets and water were provided on an ad libitum basis for 25 d from 8 to 32 d of age.

Data and sample collection

The BW and feed intake (**FI**) were measured at the conclusion of the experiment. The mortality was recorded daily. The feed conversion ratio (**FCR**) was calculated by dividing FI by BW gain (**BWG**) after adjusting the mortality (Choi et al., 2021).

Individual BW of all chickens was recorded at the end of the experiment. One male chicken, which had the closest average BW per replicated cage, was euthanized using CO_2 asphyxiation. Toe samples for the analysis of ash concentrations were collected by severing the left middle toe through the joint between the 2nd and 3rd tarsal bones from the distal end. Blood samples were collected from each bird via a heart puncture into an EDTA-tube for the analysis of heterophil to lymphocyte ratio (**H:L ratio**). Feather samples were also collected from the primary flight feather for the analysis of feather corticosterone (**CORT**) concentrations. Jejunal segments of 2–3 cm long were collected for the analysis of intestinal morphology. They were flushed with 10 % neutral-buffered formalin solution and were subsequently subjected to morphometric analysis. Jejunal mucosa samples were collected with a microscope slide and immediately frozen in liquid N_2. The collected jejunal mucosa samples were transferred and stored at –80°C before the analysis of antioxidant status and expression levels of genes in the jejunal mucosa.

Sample analysis

The experimental diets were analyzed for CP (method 984.13; AOAC, 2006) using the Kjeldahl method (Kjeltec auto system 2300 Analyzer, FOSS, Hilleroed, Denmark). An inductively coupled plasma spectrometer (Optima 5300 DV, Perkin Elmer Inc., Shelton, CT) was used to determine the concentrations of total Ca and P in diets (method 984.27; AOAC, 2006). The toe samples were dried at 100°C for 16 h and then ashed in an electric muffle furnace at 600°C for 4 h. Toe ash concentrations were determined in relation to dried toe weights (Kwon et al., 2022).

The H:L ratio in the blood was analyzed using the method of Lentfer (2015) with a minor modification. The detailed procedure was reported in our previous study (Lee et al., 2022). Feather CORT concentrations were determined using a commercial Enzo Life Sciences Corticosterone ELISA Kit (ADI-901-097; Enzo Life Sciences Inc., Farmingdale, NY), according to the method described by Lee et al. (2022).

The intestinal morphology, including villus height (**VH**), villus width (**VW**), crypt depth (**CD**), and VH:CD ratio, in the jejunum was analyzed using the method of Wiersema et al. (2021) with a minor modification. Twenty measurements were taken per jejunal sample, and their average value was calculated to represent each measurement. This analysis was performed at the BT research facility center, Chung-Ang University.

Antioxidant status, such as malondialdehyde (**MDA**), total antioxidant capacity (**TAC**), and reactive oxygen species (**ROS**) in the jejunal mucosa was determined using the following commercially available kits: OxiSelect™ TBARS Assay Kit (MDA Quantitation; STA- 330, Cell Biolabs, San Diego, CA), OxiSelect™ TAC Assay Kit (STA-360, Cell Biolabs, San Diego, CA), and OxiSelect™ In Vitro ROS/RNS Assay Kit (Green Fluorescence; STA-347, Cell Biolabs, San Diego, CA), respectively, according to the manufacturer's protocol. Protein concentrations were also analyzed using a commercial kit Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The detailed procedures for antioxidant status analysis were described in our previous study (Yu et al., 2021).

The expression levels of genes related to inflammatory cytokines, tight junction protein, and P transporters in the jejunal mucosa were analyzed according to the previous method of Kim et al. (2023) and Won et al. (2023). The primers for RT-PCR used in this study were designed using NCBI/Primer-BLAST (Table 2). The primers of the selected genes for pro-inflammatory cytokines (*IFN-γ*, *IL-1β*, and *IL-6*),

Table 2
Sequence of the primers used in real-time quantitative PCR.

RNA target	Forward	Reverse	Tm,°C	Product size (bp)	Accession no.
GAPDH	5′- TGCTGCCCAGAACATCATCC-3′	5′-ACGGCAGGTCAGGTCAACAA -3′	50–65	142	NM_204305
IFN-γ	5′-AGCTGACGGTGGACCTATTATT-3′	5′-GGCTTTGCGCTGGATTC-3′	60	259	Y07922
IL-1β	5′-GAATTCTTTGACAGTCTGCG-3′	5′-TCGGGTTGGTTGGTGATG-3′	60	244	Y15006
IL-6	5′-TCATCCTCCGAGACTTTACT-3′	5′-CCGAACTAAAACATTCAGGC-3′	58.3	100	NM_204628
IL-4	5′-ACCCAGGGCATCCAGAAG-3′	5′-CAGTGCCGGCAAGAAGTT-3′	60	258	AJ621735
IL-10	5′-CAGGGACGATGAACTTAACA-3′	5′-AGGACCTCATCTGTGTAGAA-3′	55.2	117	NM_001004414
TGF-β4	5′-CGGGACGGATGAGAAGAAC-3′	5′-CGGCCCACGTAGTAAATGAT-3′	60	258	M31160
ZO-1	5′-CACACTGTGACCCCAAAA-3′	5′-AAGGTCCATCTCAGTTTCAC-3′	56.3	151	XM_040680632
OCLN	5′-TCGTGCTGTGCATCGCCATC-3′	5′-CGCTGGTTCACCCCTCCGTA-3′	60	178	NM_205128
CLDN-1	5′-CAGACTCTAGGTTTTGCCTT-3′	5′-AATCTTTCCAGTGGCGATAC-3′	58.3	149	NM_001013611
JAM-2	5′-AATTTACAGTTCCTCCCACT-3′	5′-GTCTTTTCCAGTAAGGCAAC-3′	53.2	151	NM_001006257
MUC2	5′-CAGAAGGAACTTCTTCGTACA-3′	5′-AGAGGATGCTCCTGTAGATT-3′	53.9	137	XM_040673077
NaPi-IIb	5′-GAATTCTTTGACAGTCTGCG-3′	5′-TGCTAAGAAATGAGTAGATGGA-3′	60	174	NM_204474
PiT-1	5′-GCTCGTGGCTTCGTTCTTG-3′	5′-GGACCATTTGACGCCTTTCT-3′	58.3	109	XM_015297502
PiT-2	5′-ATGGTATCTTGCCTTACGTC-3′	5′-TAAGAAATGGCACCACATCA-3′	60	183	NM_001305398

Abbreviations: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *IFN-γ*, interferon gamma; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-4*, interleukin 4; *IL-10*, interleukin 10; *TGF-β4*, transforming growth factor beta-4; *ZO-1*, zonula occludens-1; *OCLN*, occludin; *CLDN*-1, claudin-1; *JAM-2*, junctional adhesion molecule B; *MUC2*, Mucin 2; *NaPi-IIb*, type IIb sodium-dependent phosphate co-transporter; *PiT-1*, inorganic phosphate transporter 1; *PiT-2*, inorganic phosphate transporter 2; Tm, melting temperature.

Table 3
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on growth performance in broiler chickens[a].

Treatments[b]	Growth performance				
	Final BW, g	BWG, g	FI, g	FCR, g/g	Mortality, %
Adequate P	1,813[a]	1,609[a]	2,200[a]	1.37[b]	1.7
Low P	1,796[ab]	1,592[a]	2,182[ab]	1.37[b]	3.3
Very low P	1,394[c]	1,190[c]	1,723[c]	1.45[a]	7.5
Very low P + phytase	1,704[b]	1,501[b]	2,104[b]	1.40[a]	1.7
SEM	33.3	31.4	32.4	0.016	2.1
P-value	<0.01	<0.01	<0.01	<0.01	0.17

Abbreviations: Final BW, final body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.
[a-c] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (replicated cages) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

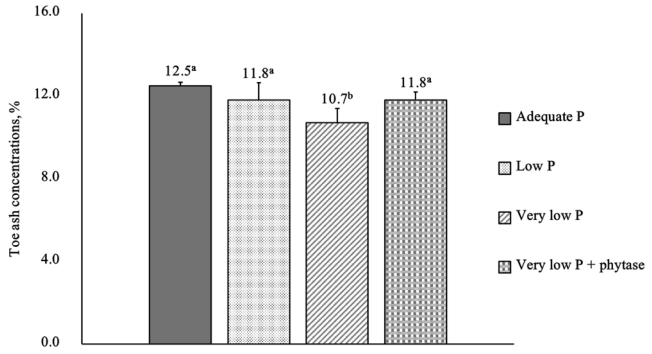


Fig. 1. Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on toe ash concentrations in broiler chickens. [a,b] Means with no common superscript differ significantly ($P < 0.05$). Data are least squares means of 10 observations (individual bird from each cage) with standard error per treatment. Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

Table 4
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on stress responses in broiler chickens[a].

Treatments[b]	Stress biomarkers	
	Blood H:L ratio	Feather CORT concentrations, ng/mg
Adequate P	0.26[b]	13.0
Low P	0.22[b]	16.4
Very low P	0.47[a]	23.8
Very low P + phytase	0.24[b]	12.8
SEM	0.032	3.12
P-value	<0.01	0.06

Abbreviations: H:L ratio, heterophil to lymphocyte ratio; CORT, corticosterone.
[a,b] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

anti-inflammatory cytokines (*IL-4, IL-10*, and *TGF-β_4*), tight junction protein (*OCLN, CLDN-1, ZO-1*, and *JAM2*), mucin (*MUC2*), and P transporters (*PiT-1, PiT-2*, and *NaPi-IIb*) were designed based on the sequences available from public databases and were synthesized by Genotech Co. Ltd. (Daejeon, Republic of Korea). The specificity of the primers was confirmed by PCR amplification. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *GAPDH* (Kim et al., 2023).

Statistical analysis

All data were analyzed as a completely randomized design by ANOVA with the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The replicated cage and the individual bird from each cage were considered the experimental unit for growth performance and other measurements, respectively. Possible outliers were checked using the UNIVARIATE procedure of SAS; however, no outliers were identified. The LSMEANS procedure was used to calculate treatment means. The PDIFF option of SAS was used to separate the means if the difference was significant. Statistical significance and tendency were set at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

Results

Performance, toe ash concentration, and stress response

Birds fed very low-P diets had less ($P < 0.05$) final BW, BWG, and FI than birds fed other treatment diets (Table 3). Moreover, birds fed very low-P diets with phytase also had less ($P < 0.05$) final BW, BWG, and FI than those fed adequate P diets. However, no differences in final BW, BWG, and FI were observed between birds fed adequate P diets and those fed low-P diets. The FCR for birds fed adequate P diets or low-P diets was less ($P < 0.05$) than for birds fed very low-P diets or very low-P diets with phytase. Toe ash concentrations were less ($P < 0.05$) for birds fed very low-P diets than for those fed other treatment diets (Fig. 1).

Birds fed very low-P diets had greater ($P < 0.05$) blood H:L ratio than those fed other treatment diets (Table 4). Similarly, a tendency ($P = 0.06$) for the greatest feather CORT concentrations was observed for birds fed very low-P diets among dietary treatments.

Jejunal morphology, antioxidant status, and gene expression

Birds fed adequate P diets or low-P diets had greater ($P < 0.05$) VH than birds fed very low-P diets or very low-P diets with phytase.

Table 5
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on jejunal morphology in broiler chickens[a].

Treatments[b]	Jejunal morphology			
	VH, μm	VW, μm	CD, μm	VH:CD ratio
Adequate P	1,384[a]	198	197	7.18
Low P	1,346[a]	190	198	6.90
Very low P	1,226[b]	191	194	6.35
Very low P + phytase	1,228[b]	185	183	6.72
SEM	40.2	9.8	8.2	0.285
P-value	0.01	0.83	0.57	0.23

Abbreviations: VH, villus height; VW, villus width; CD, crypt depth.
[a,b] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

Table 6
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on antioxidant status in the jejunal mucosa of broiler chickens[a].

	Antioxidant status		
Treatments[b]	MDA, U/mg	TAC, U/mg	ROS, mM
Adequate P	0.33[b]	538	29.8
Low P	0.58[b]	530	31.3
Very low P	0.96[a]	551	31.7
Very low P + phytase	0.38[b]	538	29.0
SEM	0.110	20.3	1.28
P-value	<0.01	0.91	0.35

Abbreviations: MDA, malondialdehyde; TAC, total antioxidant capacity; ROS, reactive oxygen species.
[a,b] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

Table 7
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on inflammatory cytokine expressions in the jejunal mucosa of broiler chickens[a].

	Pro-inflammatory cytokines			Anti-inflammatory cytokines		
Treatments[b]	*IFN-γ*	*IL-1β*	*IL-6*	*IL-4*	*IL-10*	*TGF-β4*
Adequate P	1.14	1.20	1.03	0.61[b]	0.98	0.96
Low P	1.54	1.38	1.07	1.31[a]	0.83	1.16
Very low P	0.71	0.77	1.27	0.76[ab]	1.21	1.01
Very low P + phytase	1.15	1.03	1.38	1.30[a]	0.76	0.97
SEM	0.304	0.305	0.333	0.197	0.282	0.131
P-value	0.31	0.54	0.85	0.03	0.65	0.70

Abbreviations: *IFN-γ*, interferon gamma; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-4*, interleukin 4; *IL-10*, interleukin 10; *TGF-β4*, transforming growth factor beta-4.
[a,b] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

(Table 5). However, no differences in VW, CD, and VH:CD ratio were observed among dietary treatments.

The concentrations of MDA in the jejunal mucosa were greater ($P < 0.05$) for birds fed very low-P diets than for those fed other treatment diets (Table 6). However, the concentrations of TAC and ROS in the jejunal mucosa did not differ among dietary treatments.

The expression levels of pro-inflammatory cytokine genes (i.e., *IFN-γ, IL-1β*, and *IL-6*) in the jejunal mucosa were not affected by dietary treatments (Table 7). However, regarding anti-inflammatory cytokine genes (i.e., *IL-4, IL-10*, and *TGF-β4*), the expression levels of *IL-4* were greater ($P < 0.05$) for birds fed low-P diets or very low-P diets with phytase than for birds fed adequate P diets, whereas expression levels of *IL-4* for birds fed very low-P diets did not differ from birds fed other treatment diets. However, expression levels of *IL-10* and *TGF-β4* were not influenced by dietary treatments.

For tight junction-related genes (i.e., *ZO-1, OCLN, CLDN-1*, and *JAM-2*) in the jejunal mucosa, the expression levels of *OCLN* for birds fed low-P diets or very low-P diets with phytase were greater ($P < 0.05$) than for birds fed adequate P diets (Table 8). Birds fed low-P diets exhibited

Table 8
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on the expression levels of genes related to tight junction and mucus in the jejunal mucosa of broiler chickens[a].

	Tight junction-related genes				Mucus gene
Treatments[b]	*ZO-1*	*OCLN*	*CLDN-1*	*JAM-2*	*MUC2*
Adequate P	0.90	0.69[c]	0.96	1.29	0.83
Low P	1.39	1.29[a]	1.18	1.43	1.42
Very low P	0.97	0.86[bc]	1.16	1.85	0.65
Very low P + phytase	1.70	1.27[ab]	1.27	1.33	1.38
SEM	0.185	0.147	0.117	0.365	0.240
P-value	0.08	0.01	0.50	0.69	0.05

Abbreviations: *ZO-1*, zonula occludens-1; *OCLN*, occludin; *CLDN-1*, claudin-1; *JAM-2*, junctional adhesion molecule B; *MUC2*, Mucin 2.
[a-c] Means within a column with no common superscript differ significantly ($P < 0.05$).
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

Table 9
Effect of decreasing phosphorus (P) concentrations in diets and phytase supplementation on the expression levels of P transporter genes in the jejunal mucosa of broiler chickens[a].

	P transporter genes		
Treatments[b]	*NaPi-IIb*	*PiT-1*	*PiT-2*
Adequate P	0.63	0.96	0.98
Low P	1.33	0.97	1.31
Very low P	1.04	0.86	1.24
Very low P + phytase	1.15	1.10	1.42
SEM	0.251	0.171	0.144
P-value	0.25	0.78	0.18

Abbreviations: *NaPi-IIb*, type IIb sodium-dependent phosphate cotransporter; *PiT-1*, inorganic phosphate transporter 1; *PiT-2*, inorganic phosphate transporter 2.
[a] Data are least squares means of 10 observations (individual bird from each cage) per treatment.
[b] Adequate P = 0.44 % available P; Low P = 0.30 % available P; Very low P = 0.10 % available P; Very low P + phytase = very low-P diets supplemented with 1,000 FTU/kg phytase (assumed P equivalency value for 1,000 FTU/kg phytase is 0.20 % available P).

greater ($P < 0.05$) expression levels of *OCLN* than birds fed very low-P diets. Likewise, there was a tendency ($P = 0.08$) for greater expression levels of *ZO-1* in the jejunal mucosa of birds fed low-P diets or very low-P diets with phytase than birds fed adequate P diets or very low-P diets. However, expression levels of other 2 tight junction-related genes (*CLDN-1* and *JAM-2*) were not affected by dietary treatments. Birds fed low-P diets or very low-P diets with phytase showed a tendency ($P = 0.05$) for greater expression levels of *MUC2* gene than those fed adequate P diets or very low-P diets.

The expression levels of P transporter genes (i.e., *NaPi-IIb, PiT-1*, and *PiT-2*) in the jejunal mucosa were not influenced by dietary treatments (Table 9). However, the expression levels of *NaPi-IIb* and *PiT-2* genes for birds fed adequate P diets were numerically less than for birds fed other treatment diets.

Discussion

Dietary P deficiency exerts a negative impact on productive performance in broiler chickens. Wang et al. (2020) reported that decreasing concentrations of aP from 0.34 % to 0.13 % in diets linearly decreased growth performance in broiler chickens during 7 to 21 d of age.

Adedokun et al. (2004) also reported growth depression in broiler chickens fed diets with reduced aP concentrations (0.12 %) compared with those fed diets with recommended aP concentrations (0.45 %) during 8 to 22 d of age. A similar reduction in broiler performance by feeding very low-P diets (0.10 % aP) was observed in this study.

Comparing with high P deficiency (i.e., very low-P in diets), however, previous studies reported that a moderate decrease in dietary P concentrations showed little negative impact on growth performance in broiler chickens. Valable et al. (2018) reported no differences in growth performance between broiler chickens fed diets containing 0.40 % aP and those fed diets containing 0.30 % aP during 11 to 21 d of age. Hamdi et al. (2015) also observed no differences in broiler performance by feeding diets containing 0.31 % aP as compared to recommended levels of aP during 7 to 14 d of age. These results are consistent with our observations for birds fed low-P diets. Therefore, it may be suggested that the moderate reduction of up to 0.30 % aP level in broiler diets can maintain broiler performance in a manner similar to recommended levels of dietary aP (0.44 % aP). This observation may indicate that recommended levels of dietary aP in nutritional standards of the broiler breeder exceed the requirement of dietary aP for broiler performance, which is possibly attributed to the consideration of safety margins for dietary nutrient supply.

Dietary phytase is known to hydrolyze dietary phytic acid into myo-inositol and inorganic phosphate in the gastrointestinal tract (**GIT**) of animals (Wang et al., 2020). Therefore, dietary phytase is typically supplemented to low-P diets for poultry with its expected matrix values (i.e., equivalency values) of aP. This practice has been reported to successfully maintain growth performance in broiler chickens (Selle and Ravindran, 2007; Simons et al., 1990; Wang et al., 2020). However, in this study, phytase supplementation in very low-P diets exhibited decreased broiler performance although the concentrations of aP in very low-P diets was calculated using the matrix value of supplemental phytase (i.e., 1,000 FTU/kg = 0.20 % aP) and those were equal to aP concentrations in low-P diets. This result may indicate that phytase supplementation in very low-P diets cannot fully support normal growth performance in broiler chickens, despite the application of expected matrix values of aP for phytase to feed formulations. One possible reason may be that the matrix values of aP for phytase are generally determined based on bone characteristics rather than growth performance in broiler chickens, thereby limiting the prediction of broiler performance. Moreover, it should be emphasized that the matrix values of aP for specific phytase determined under one study may be difficult to be adopted in other studies because large variations in experimental conditions exist among studies (Adeola and Cowieson, 2011).

Feeding very low-P diets to broiler chickens decreased toe ash concentrations in this study, revealing that the current aP levels in very low-P diets were largely deficient for bone development in broiler chickens. Adedokun et al. (2004) and Liu et al. (2017) also reported a decrease in toe ash concentrations by feeding very low-P diets to broiler chickens. However, previous studies also demonstrated that feeding diets containing moderately low aP concentrations decreased toe ash concentrations in broiler chickens. Liu et al. (2017) reported that toe ash concentrations were decreased with reduced aP levels from 0.43 % to 0.33 % in broiler diets during the first 21 d of age. Likewise, Perney et al. (1993) also observed a reduction in toe ash concentrations by decreasing levels of aP from 0.45 % to 0.29 % in broiler diets during 2 to 16 d of age. However, these results contradict our findings that feeding low-P diets had no negative effects on toe ash concentrations in broiler chickens. This observation may be attributed to the fact that one-phase low-P diets containing 0.30 % aP were fed to broiler chickens for overall experimental periods (i.e., 8–32 d of age) in this study, such that it may increase dietary aP supply closer to the recommended levels of aP for broiler chickens during finishing periods, thereby mitigating the reduction in toe ash concentrations by feeding low-P diets. Interestingly, similar toe ash concentrations were identified between birds fed low-P diets and those fed very low-P diets with phytase, indicating that the matrix values of aP for phytase adopted in this study was appropriate to predict toe ash concentrations in broiler chickens. The reason may be that the matrix values of aP for phytase used in this study were established using bone ash concentrations under similar experimental conditions (Kwon et al., 2022).

Blood H:L ratio and feather CORT concentrations have been widely used to assess stress responses in poultry (Lee et al., 2022). However, information regarding stress responses in poultry by feeding diets with nutritional deficiencies is currently lacking. One study reported that reduced levels of aP by 20 % in diets from recommended levels of aP increased blood H:L ratio in broiler chickens (Nari et al., 2020). Similarly, Park et al. (2024) reported that feeding very low-P diets (0.20 % aP) to laying hens increased CORT concentrations in egg yolk as compared to feeding adequate P diets (0.40 % aP). In the present study, we also found that feeding very low-P diets to broiler chickens increased blood H:L ratio and feather CORT concentrations compared with feeding adequate P diets. This result suggests that dietary P deficiency enhances stress responses in broiler chickens. The possible reason may be associated with impaired gut homeostasis by dietary P deficiency, which leads to an activation in gut-brain axis inducing stress responses (Mumma et al., 2006; Park et al., 2024). However, no differences in blood H:L ratio and feather CORT concentrations were observed among birds fed adequate P diets, low-P diets, or very low-P diets with phytase in this study. Similar results for no increase in stress responses were also observed by feeding low-P diets or very low-P diets with phytase to laying hens (Park et al., 2024). Therefore, these findings suggest that the moderate reduction in dietary P concentrations may not largely induce stress responses in poultry.

Decreased P concentrations in diets were reported to have a negative effect on intestinal morphology in poultry (Emami et al., 2013, Hezaveh et al., 2020). In the present study, jejunal VH was lower for birds fed very low-P diets than for birds fed adequate P diets or low-P diets, which agrees with the previous findings. However, no differences in VH were observed between birds fed adequate P diets and those fed low-P diets despite different aP concentrations between 2 treatment diets. Nari et al. (2020) also reported no significant changes in jejunal VH by reducing levels of aP by 20 % from recommended levels of aP in broiler diets. Therefore, the reason for no differences in jejunal VH as observed between birds fed adequate P diets and those fed low-P diets may be that one-phase diets were fed to both growing and finishing chickens in this study; therefore, the degree of reduced P concentrations in low-P diets was insufficient to exert a negative impact on the jejunal morphology. Dietary phytase can increase aP levels in diets by hydrolyzing organic phytate to inorganic P. It was expected, therefore, that decreased intestinal VH by decreasing P concentrations in diets can be ameliorated by dietary phytase supplementation in poultry (Emami et al., 2013, Hezaveh et al., 2020, Nari et al., 2020). However, we did not detect such a positive effect on jejunal VH in this study. Pirgozliev et al. (2008) reported that phytase supplementation in low-P diets had no effects on ileal VH in broiler chickens. The reason for the failure to maintain jejunal VH by phytase supplementation in very low-P diets is unclear; however, it may be involved in an improvement in Ca absorption and utilization. Dietary phytase is known to increase both dietary Ca and P utilization (Adeola and Cowieson, 2011), such that phytase supplementation may induce an imbalance in dietary Ca:aP ratio, leading to an impairment in morphological development (Aptekmann et al. 2001). However, decreasing aP concentrations in diets and phytase supplementation in very low-P diets appear to exert no negative impacts on VW, CD, and VH:CD ratio in broiler chickens.

Increased MDA concentrations in the jejunal mucosa of broiler chickens were observed by feeding very low-P diets, whereas no such an effect was noted by feeding low-P diets or very low-P diets with phytase in this study. Chen et al. (2017) reported that dietary P deficiency may accelerate lipid peroxidation by decreasing antioxidant systems such as depressed glutathione synthesis and downregulated expression of antioxidant enzymes in fish. Alagawany et al. (2020) reported that feeding

low-P diets increased serum MDA concentrations in Egyptian geese. However, Zhang et al. (2020) observed no changes in serum MDA concentrations by decreasing aP concentrations in broiler diets, which agreed with the current observation for birds fed low-P diets. Interestingly, we found that jejunal MDA concentrations in birds fed very low-P diets with phytase were numerically less than in birds fed low-P diets, and even closer to those fed adequate P diets. Previous studies have reported that dietary phytase supplementation improves antioxidant capacity in broiler chickens (Karadas et al., 2010; Liu et al., 2010), possibly owing to up-regulated antioxidant enzyme activity as a consequence of increased phytate degradation and inositol release by phytase (Lee et al., 2019). However, no effects of decreasing aP concentrations in diets and phytase supplementation in very low-P diets on other antioxidant indicators, including TAC and ROS concentrations, in the jejunal mucosa were identified in this study. The reason for these inconsistent results is unknown; however, antioxidant measurements of MDA, TAC, and ROS levels in body tissues may not be closely correlated. Gao et al. (2013) suggested that antioxidant systems may work differently in various body tissues due to different sources or levels of oxidative agents. In addition, the intestinal mucosa contains abundant phospholipids, possibly explaining the significant changes in MDA production with negligible relation to TAC and ROS levels (Breganó et al., 2009).

Inflammatory cytokines serve an important role in regulating innate and adaptive immune functions, with the balance of pro-inflammatory and anti-inflammatory cytokines being critical for the pathological inflammatory status (Kidd, 2003; Sultani et al., 2012). Several studies have investigated inflammatory responses to decreasing concentrations of dietary P in poultry. Bai et al. (2015) found that laying hens fed diets containing deficient levels of aP exhibited increased expression of *IL-4* in cecal tonsils than those fed adequate P diets, with no alterations in expression levels of *IFN-γ, IL-1β, IL-6*, and *IL-10*. Rodriguez-Lecompte et al. (2016) observed no changes in expression levels of *IFN-γ, IL-4*, and *IL-10* in peripheral blood mononuclears by feeding Ca and P deficient diets; nevertheless, they found increased expression levels of these genes in both spleen and bursa of fabricious in broiler chickens. Nie et al. (2018) reported that serum concentrations of *IFN-γ* were decreased by feeding low-P diets to laying hens without affecting *IL-1β* and *IL-6* expressions. In the current study, no obvious changes in both pro-inflammatory and anti-inflammatory cytokines in the jejunal mucosa were detected among dietary treatments, except for *IL-4*. The expression levels of *IL-4* in the jejunal mucosa were increased by feeding low-P diets or very low-P diets with phytase as compared to feeding adequate P diets. *IL-4* is considered an anti-inflammatory cytokine to alleviate inflammatory immune responses (Bai et al., 2015); therefore, moderate reduction in dietary aP concentrations may suppress inflammatory immune responses in the jejunal mucosa. However, it is difficult to explain why the expression levels of other pro-inflammatory and anti-inflammatory cytokines were not altered by decreasing aP concentrations in diets and phytase supplementation in very low-P diets in this study.

The absorption of P in the GIT of animals is mediated by transcellular and paracellular routes (Adedokun and Adeola, 2013). Although the paracellular transport is partly responsible for intestinal P absorption, the transcellular transport is recognized as the primary pathway of intestinal P absorption (Adedokun and Adeola, 2013). The major P transporters in the GIT of poultry include *NaPi-IIb, PiT-1*, and *PiT-2* (Fang et al., 2012; Hu et al., 2018). Previous studies have revealed that intestinal expression levels of P transporter genes may depend on dietary P intake. Hu et al. (2018) reported that expression levels of *PiT-1* and *NaPi-IIb* in the jejunum were increased by feeding low-P diets to broiler chickens at 7 and 21 d of age. Similar results were also obtained by Aderibigbe et al. (2022) and Omara et al. (2020) who reported that decreasing aP concentrations in diets increased expression levels of *NaPi-IIb* in the duodenum and jejunum of broiler chickens at 14 and 18 d of age. The possible reason has been associated with the fact that chickens fed low-P diets attempt to increase P absorption by increasing expression of P transporting systems in the GIT (Aderibigbe et al., 2022; Hu et al., 2018; Omara et al., 2020). However, expression levels of *NaPi-IIb, PiT-1*, and *PiT-2* were not affected by decreasing aP conentrations in diets and phytase supplementation in very low-P diets in this study, despite insignificant increase in expression levels of *NaPi-IIb* and *PiT-2* being found by feeding diets containing decreasing aP concentrations. The reason for this observation is unclear; however, insignificant increase in expression levels of 2 specific P transporter genes may be related to increasing length and size of small intestine. Park et al. (2024) reported that feeding low-P diets increased the relative weight of small intestine in pullets, suggesting a compensatory increase in the volume of small intestine for increasing P absorption in response to reduced P supply in diets. However, there are still variable results for P transporter expressions in response to decreasing P concentrations in diets, which may be attributed to not only different functions of each P transporter in varying sections of the GIT, but also variations in the degree and duration of feeding low-P diets among studies. Moreover, it should be noted that this study only measured mRNA expression levels of 3 selected P transporter genes (e.g., *NaPi-IIb, PiT-1*, and *PiT-2*), which may limit clear explanation for the effects of decreasing concentrations of dietary P on intestinal P transporting systems. Further research is required to study both protein expression of more P transporters and direct transporting efficiency of dietary P in intestinal tissues of broiler chickens as influenced by decreasing P concentrations in diets.

Conclusion

The current study hypothesized that decreasing P concentrations in diets may enhance stress response and impair intestinal health in broiler chickens and phytase supplementation in very low-P diets may alleviate these negative outcomes. Feeding very low-P diets (0.10 % aP) impaired growth performance, bone development, and jejunal VH in broiler chickens with enhancing stress response and intestinal lipid peroxidation. However, the moderate reduction in dietary P concentrations (0.30 % aP) from adequate P levels (0.44 % aP) exerted no negative impact on growth performance, bone development, stress responses, intestinal morphology, and antioxidant status in broiler chickens. Likewise, feeding very low-P diets with phytase maintained bone development, stress responses, and antioxidant status as observed in broiler chickens fed adequate P diets, but decreased growth performance and jejunal VH. Finally, no apparent modifications of gene expression levels of intestinal P transporters were identified by decreasing P concentrations in diets and phytase supplementation in very low-P diets under the current experimental condition.

Disclosures

The authors declare no conflict of interest for the data presented in this experiment.

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